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Generation of murine bone-marrow derived macrophages and using tumor co-culture assays to mimic tumor microenvironment

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Abstract

Macrophages are one of the key immune cells within the tumor microenvironment that encourage the growth of tumors at the primary site as well as contributing to all parts of the metastatic cascade. Although it is possible to isolate macrophages directly, this can be a laborious process and due to their plasticity, it is not possible to maintain their *in vivo* phenotype while *in vitro*. For this reason, differentiating macrophages from bone marrow is an attractive alternative. Here we present robust methods to study *in vitro* derived macrophages including (i) the isolation and generation of macrophages from bone marrow, (ii) differentiation/characterization of classically activated (anti-tumoral), alternatively activated (pro-tumoral) and tumor-conditioned macrophages, as well as (iii) *in vitro* co-culturing assays for tumor cell-macrophage interaction/transmigration.

1. Introduction

Macrophages *in vivo* can be derived from bone marrow (BM) derived monocytes, fetal liver and from yolk-sac-progenitors. They are usually defined as mononuclear phagocytic cells that reside in all tissues and are involved in a wide range of processes such as in immune responses and in the regulation of tissue growth and homeostasis (Geissmann et al., 2010; Pollard, 2009). In addition, they are involved in almost all pathologies either in their resolution or in their acerbation (Wynn, Chawla, & Pollard, 2013). In the context of this series, macrophages have been shown in most cases to exert pro-tumoral effects within the tumor microenvironment (De Palma & Lewis, 2013; Cassetta & Pollard, 2018; Mantovani, Marchesi, Malesci, Laghi, & Allavena, 2017). Macrophages in the tumor microenvironment show a different phenotype to inflammatory and homeostatic cells (Qian & Pollard, 2010) and exert functions that enhance cancer malignancy at every step of the metastatic cascade. These functions include the induction of angiogenesis, enhancement of tumor cell dissemination to metastatic sites where they also enhance extravasation and metastatic cell survival and growth (Kitamura, Qian, & Pollard, 2015). They also produce an immunosuppressive microenvironment which contributes to immunotherapy/chemotherapy resistance (Cassetta & Pollard, 2018; DeNardo & Coussens, 2007). The link between poor prognosis and high macrophage infiltration has also been established in many but not all human cancers (Jochems & Schlom, 2011; Yang, McKay, Pollard, & Lewis, 2018). Besides pro-tumoral macrophages, the release of inflammatory factors in the tumor microenvironment such as IFN-γ, could activate tumoricidal macrophages that enhance an anti-tumor immune response however the persistence of this phenotype within an immunosuppressive tumor microenvironment is unclear (Mantovani & Sica, 2010). Recent studies have shown

that macrophages can be therapeutically be reprogrammed using agonistic or antagonistic drugs and thereby be diverted to fight against cancer. (Cassetta & Pollard 2018).

In mice, bone marrow derived $CD11b^{+}CD11c^{-}Ly6G^{-}Ly6C^{high/low}$ circulatory monocytes are recruited to the tissue and differentiate into $Ly6C^{-}F4/80^{+}CD11b^{+}$ macrophages. These cells populate inflammatory tissue and are present in large numbers in the tumor microenvironment (Epelman, Lavine, & Randolph, 2014) as well as many tissues during homeostasis such as the intestine, replacing yolk sac derived macrophages. Macrophages are highly versatile cells that display a wide spectrum of phenotypes. Despite this fact they are often over-simplistically classified as classically activated macrophages that play an immune effector/anti-tumoral role, and alternatively activated macrophages that play an immunoregulatory/pro-tumoral role (Gordon & Taylor, 2005; Mosser & Edwards, 2008) This nomenclature has also been superimposed upon the classification of “M1 or M2’ based observations in different mouse strains with different susceptibilities to cancer (Mantovani & Sica, 2010) to reflect Th1 and Th2 responses in vivo. However, this classification is more representative of *in vitro* derived macrophages using cytokine-mediated differentiation rather than their phenotype of in vivo. To clarify these responses, Murray and colleagues suggest a nomenclature that links to the cytokines used for the activation such as M(IL-4), M(IL-10) (Murray et al., 2014) at least in vitro. But the situation in vivo is likely to represent a much more mixed phenotype given the plethora of signals such cells respond to (Cassetta et al., 2019).

Although it is possible to sort macrophages from digested tissues, samples can be scarce, and this can be a time consuming and expensive process with high variability

between subjects/animals. Consequently, the development of means to isolate macrophages from bone marrow originally perfected by Stanley (Stanley, 1985, 1997; Tushinski et al., 1982) has provided a boon to studies of macrophage biology. This is because large numbers of cells at greater than 95% purity can be obtained from a small number of mice (approximately 10-15 million bone marrow cells can be isolated from a mouse femur) and the isolation protocol and differentiation is straight-forward and cost-effective. These cells respond by proliferation to the growth factors Colony stimulating factor (CSF)1, CSF2 and IL-3 and they can be polarized *in vitro* with a wide range of factors. At least 95-98% of these cells are dependent on CSF1 for viability (Stanley, 1985; Tushinski et al., 1982). Furthermore, the ability to isolate them from any mutant mouse that contains a null or conditional allele of any gene of interest allows studies on growth factor signalling and macrophage function. In addition, there are studies where these bone-marrow derived macrophages (BMDMs) or their conditioned medium (CM) can be transferred to recipient mice and functions studied *in vivo* (Saha et al., 2016; Thomas et al., 2011).

Although BMDM isolation is a widely used protocol for *in vitro* macrophage studies there is a diversity of methods for their maturation and differentiation. Therefore, in this chapter we aim to demonstrate a standard BMDM isolation, methods for differentiation with cytokines/growth factors and for studying the roles of macrophages in tumor biology.

2. Isolation and Differentiation of Murine BMDM

2.1 Introduction

BMDM isolation shows methodological differences among various studies but as yet it is unclear whether those differences affect functional outcome. Some important steps in this protocol should be noted to reduce the variation among different isolation methods. For example, to increase the purity of hematopoietic stem cell isolation, we used tissue culture plates on the first day of isolation to allow mesenchymal stem cells, fibroblasts, mature macrophages and other adherent cells to attach but allow hematopoietic cells to be free within the medium. We then transfer the cell suspension into a low adhesive petri plate to continue their differentiation. In addition, different BMDM seeding densities are used among various methods and it has been shown that higher seeding densities might affect macrophage phenotype (Lee & Hu, 2013). In this protocol, seeding $3x10^6$ cell per $10cm^2$ dish gave a pure $Ly6C^-Ly6G^-CD11b^+F4/80^+$ macrophage population as shown in Figure 1. Both human and mouse recombinant CSF1 (historically often called macrophage-CSF, M-CSF) can be used for differentiation. Human recombinant CSF1 has been shown to be fully effective for mouse BMDM differentiation (Stanley, 1985, 1997). We analysed BMDM phenotype on day 8, although BMDMs can be used for up to 3 weeks after differentiation as previously reported (Davies & Gordon, 2005). They can also be frozen down for use later.

2.2 Collection of Mouse Femur and Tibia

1. Euthanize the mouse according the approved animal license procedure.
2. Place the mouse in supine position and disinfect the legs and the hips by spraying with 70% (v/v) Ethanol.

3. Using scissors cut the skin along the inguinal area and pull back the entire leg skin.
4. Remove the quadriceps and the tibias anterior muscle with forceps or a blade, until femur and tibia bone is visible.
5. Using forceps twist the femur and dislocate the femoral head from the hip with scissors. Make sure the femur is not damaged.

   Note: Bones could be stored or shipped in 45 ml of fetal bovine serum free DMEM medium supplemented with 1% (v/v) Penicilin Streptomycin at 4°C for 1-3 days. The effect of storage on BMDM isolation is characterized by Papazian et al. (Papazian, Kfoury, Scadden, 2017)
6. After the femur is completely dislocated from the cadaver, using forceps twist the knee joint between femur and tibia. Cut the knee joint and connective tissue. Make sure the tibia is not damaged.
7. Repeat the same procedure to dislocate distal tibia from talus.
8. Remove the excess muscle and connective tissue using sterile 70% ethanol-soaked tissue wipes and a blade.
9. Place the femurs and tibias on a petri dish and place them on ice.

2.3 Bone Marrow Isolation

1. Under the sterile laminar flow hood, hold the femur or tibia using forceps and gently cut the tip of the proximal and distal epiphysis of the bone. Make sure metaphysis of two edges are exposed.
2. Hold the bone in a vertical position and gently insert a $27^{1/2}$G needle from the proximal metaphysis. Using a 5ml syringe, flush 5ml of cold phosphate buffered

saline (PBS), (without calcium chloride or magnesium chloride, pH 7.4) into a 50ml empty falcon tube.

3. Rotate the bone upside down and repeat the same procedure from the distal metaphysis side.
4. Using a 1 ml pipette, mix the bone marrow cell suspension to homogenize into a single cell suspension.
5. Pass the suspension through a 70µm cell strainer to remove any bone fragments and blood clots.
6. By using the cap of a 1ml plunger, homogenize any bone marrow fragments that remain on 70µm cell strainer. Wash the filter with 5ml more PBS.
7. Centrifuge at 400 g for 5 min at 4°C.
8. Discard the supernatant and re-suspend the pellet in 30mL of PBS.
9. Take 20µl of homogenized cell suspension and count the cell number using manual or automated cell counter.
10. Centrifuge at 400g for 5 min at 4°C.
11. Discard the supernatant.

2.4 BMDM maturation and differentiation

1. Re-suspend the cells with alpha-MEM medium (Gibco, Cat #: 12571063) supplemented with 10% v/v fetal bovine serum (FBS), (Gibco, Cat #: 11573397) and 1% v/v penicillin streptomycin (PS) (Gibco, Cat #: 15140122) at a concentration of $3x10^5$ cell/ml.
2. Split 10 ml of cell suspension to each tissue culture treated $10cm^2$ plate.
3. Add 4.4nM (10^4U/ml) recombinant CSF1 to each plate.

4. Incubate cells in a humidified incubator with 5% CO_2 at 37°C
5. 24h later, transfer non-adherent cells into a non-tissue culture treated petri dish.

   Note: This procedure is required to remove adherent mesenchymal stem cells.
6. Replace the medium and 4.4nM (10^4U/ml) of recombinant CSF1 every 2-3 days.

   Note: CSF1 consumption rate/cell density is described in detail by Tushinski et al. (Tushinski et al., 1982)
7. For the first 3 days cells will not be adherent, therefore add fresh CSF1 and top up to 10ml final volume.

   Note: Bone marrow cells that are in 3-4 days in culture could be stored in 10% v/v DMSO FBS solution in -196°C for the long term.
8. For subsequent days aspirate the media and add fresh CSF1 and medium to adherent cells on the plate.
9. On Day 7 collect the cells for analysis or for differentiation.
10. To collect cells, place the cell culture plate on ice, aspirate the medium and wash the plates with ice cold PBS. Add 5mL alpha-MEM medium and using a cell scraper gently scrape the bottom of the plate and collect the macrophages.
11. Collect the cell suspension into a 50mL falcon tube, centrifuge at 400g for 5 min at 4°C.
12. Process cells for analysis or seed $2x10^5$ cells per 6-well plate with 3ml alpha-MEM full medium with 4.4nM CSF1 for differentiation.
13. To differentiate them into different macrophage subtypes, M(IFNγ+LPS), M(IL4+IL13) and tumor conditioned macrophages (TCM) use the reagent mix shown in Table 1 and Table 2.

Note: Tumor-conditioned medium (CM) can be collected from any murine or human tumor cell line, not forgetting species difference in response to factors. In this assay we used the murine mammary carcinoma E0771 cell line. Briefly, seed 1x10^6 tumor cells into 75cm^2 flask using DMEM medium supplemented with 10% v/v FBS. 24h later when cells are 70-80% confluent, collect the medium and centrifuge at 400g for 5 minutes. Filter the medium using 0.44µm low protein binding syringe filter. Transfer the supernatant into a new Falcon tube. Use the CM fresh or freeze in aliquots at -80°C. Use 20% v/v of this medium for BMDMs differentiation.

14. 24h after differentiation aspirate the medium and wash the plates with PBS.
15. Aspirate PBS and add 1ml of Accutase® (Sigma-Aldrich, Cat # A6964) to each well.

    Note: Because differentiated cells are more adherent, accutase will help cells to detach from the plate.
16. Incubate cells for 15-20 min at 37°C.
17. Place the cell culture plate on ice and using a cell scraper gently scrape the bottom of the plate and collect macrophages.

3. **Characterization of mouse BMDM phenotype**

3.1 Introduction

While BMDMs are an incredibly useful model to study macrophage function *in vitro*, for some of the reasons mentioned above, it's important to establish standard characterization methods to reduce the variability between different *in vitro* experiments and to validate their phenotypic similarities to *in vivo* macrophage subtypes.

There are a variety of ways to do this one of which is flow cytometry. Flow cytometry is a convenient and effective method allowing a researcher to accurately and simultaneously analyze several cell surface and intracellular markers by immunolabeling cells, allowing the identification of sub-populations that may exist within the BMDM preparation (e.g. mesenchymal stem cells, dendritic cells etc.), while gating out any other cell population of interest that could be co-cultured with BMDM.

It is important to select markers that successfully distinguish different macrophage subtypes clearly. There are a number of cell surface markers that could be used for phenotypic characterization using flow cytometry. With no exception they will all show $CSF1R^{+}$ (CD115) and F4/80 positivity (Table 3). To identify different subtypes here we have selected CD86, CD206 and MHC-II markers. CD86 is a co-stimulatory molecule that is the most upregulated in classically activated macrophages (Gensel, Kopper, Zhang, Orr, & Bailey, 2017) or M(IFNγ+LPS) and successfully distinguishes it from both alternatively activated macrophages or M(IL4+IL13) and those treated with TCM (Figure 1C). CD206 is a mannose receptor that is upregulated on M(IL4+IL13) (Stein et al., 1992) and TCM (Figure 1C). As well as these markers, MHC-II can be used in combination with the above to distinguish TCM from the other macrophage subtypes.

For example, M(IFNγ+LPS) are $CD86^{hi}$, $CD206^{lo}$, $MHC\text{-}II^{hi}$ while M(IL4+IL13) are $CD86^{lo}$, $CD206^{hi}$ and $MHC\text{-}II^{lo}$ and TCMs are $CD86^{lo}$, $CD206^{hi}$ and $MHC\text{-}II^{hi}$.

3.2 Staining and controls

1. Collect cells from petri plate as described in 2.4. Centrifuge them at 400g for 5 mins at 4°C.
2. Discard supernatant and homogenize pellet with 5mL of flow cytometry buffer (5% w/v bovine serum albumin in 1x PBS, pH 7.4).
3. Centrifuge at 400g for 5 mins at 4°C.
4. Discard supernatant and resuspend with flow cytometry buffer on ice.
5. Pass suspension through 70µm cell strainer to ensure a single cell suspension.
6. Count cells and aliquot 0.5-1x10^6cells/100µL to flow cytometry tubes on ice-label appropriately (Samples and controls listed in Table 4)
7. Add 1µl of Fc block™ (CD16/CD32) (BD Pharmingen, Cat #: 564219) per 1x10^6cells on ice for 10 mins.
8. Add antibody cocktail (Table 4-5) and incubate on ice for 30-60mins.
9. Add 1-2mL flow cytometry buffer to each tube and centrifuge at 400g for 5 mins at 4°C. Discard the supernatant and repeat the wash step one more time.
10. Resuspend in 300µL flow cytometry buffer and add 0.3µl of 100µg/ml DAPI before running the samples on flow cytometer. Make sure samples are thoroughly mixed.

3.3 Data acquisition and gating strategy (DIVA software, BD 6laser-LSR)

1. Acquire unstained cells to set up the correct voltages for forward scatter (FSC) and side scatter (SSC). Make sure all cells are on scale and the main cell population is distinct from cell debris.
2. Prior to fluorescence compensation setup, run an unstained and a fully stained sample to make sure that none of the colors are off-scale. The unstained histogram should be around 10^2 in logarithmic scale and fully stained should be under 10^5.
3. Run compensation beads (eBioscience) stained with a single antibody. This will result in a proportion of stained and unstained beads. Follow the software's usual method for compensation.
4. Start acquiring your samples. When acquiring data, set the 'stopping gate' to the $CD11b^{+,}$ $F4/80^{+}$ gate (See Figure 1A) and collect at least 10,000 events within this gate. These are considered the macrophages. These gates can be set up by gating on positive cells compared to their respective FMO.
5. Save all of your data onto appropriate software/hardware for further analysis on your preferred analysis software.

4. BMDM Cancer Cell Co-culture Assays

4.1 Introduction

Macrophages induce tumor cell progression, survival and migration through reciprocal cell-cell interactions *in vivo*. Several of these activities can be mimicked in cell culture thus allowing exploration of mechanism that in turn can be re-explored within *in vivo* models. We have performed a number of these assays over the years describing for example, a paracrine loop between TAMs and tumor cells involving tumor synthesized CSF1 and macrophage produced EGF ligands (Condeelis & Pollard, 2006). Most

recently we have studied the mechanism whereby macrophages promote metastasis via their stimulation of extravasation and tumor cell survival at metastatic sites (Kitamura & Pollard, 2015). To this end we developed relatively simple assays involving two-or three-cell types that have high fidelity when translated back *in vivo*. These assays allow screening using mutant BMDM and inhibitors to establish function. One such assay we use is the "*In vitro* transendothelial migration" (eTEM) assay which is designed to mimic the extravasation of tumor cells and thus combines tumor and endothelial cells with macrophages. A similar assay was also developed for intravasation (Wyckoff et al., 2004). For the eTEM assay macrophages are seeded on the underside of a transwell, while the upper-side is coated with matrix and endothelial cells. Cancer cell extravasation from the apical side of endothelial cells to the macrophage rich underside is quantified. This assay has previously been used to demonstrate that inflammatory monocytes as well as VEGF and CCL3 expression in metastasis-associated macrophages promotes cancer cell transmigration (Kitamura et al., 2015; Qian et al., 2011). Time-lapse imaging is a second method used in the same study to demonstrate tumor cell-macrophage cell-cell interactions and thus showed that CCR1 expression is required by macrophages to interact with cancer cells (Kitamura et al., 2015). Below is a detailed description of these two assays. It should be acknowledged that there are many other types of assays published that look at tumor cell-macrophage interactions often using sophisticated microfluidic devices see for example (Zervantonakis et al., 2012) or spheroid type assays. Readers are referred to this large literature to select the assay most suitable to their needs.

4.2 In vitro Transendothelial Migration Assay

1. *2 days before the experiment:* For migration assays use a growth factor reduced matrigel coated 8µm pore size transwell insert (Corning, Cat # 354483). Thaw the inserts at room temperature for about 10min.
2. Preincubate the transwells with DMEM (Corning, Cat # 10-013CVR) medium and 10% v/v FBS by adding 300µl on upper chamber and 500µl on bottom chamber. Incubate at 37°C for 2 hours.
3. Preparation of 3B-11 cells (SV40 transformed mouse endothelial cell line):
    a. Culture 3B-11 cells in DMEM medium supplemented with 10% v/v FBS.
    b. Aspirate the medium and wash cells with 1x PBS. Aspirate the buffer
    c. Add 3ml Trypsin-EDTA (0.25%) (Gibco) on T75cm^2 flask
    d. Incubate 5min at 37°C. Stop the enzymatic process by adding 3ml of DMEM complete medium. Collect detached cells into a 15ml falcon tube
    e. Centrifuge cells at 400g for 5min. Discard the supernatant.
    f. Resuspend cells in DMEM complete medium. Adjust the cell concentration to 10^5 cells per/ml.
4. Aspirate the medium on both sides of the transwells. Make sure matrigel and membrane is not damaged.
5. Add 200µl of the 3B-11 cells suspension on top of the matrigel and add 750µl DMEM medium on the bottom chamber. Incubate cells for 48h at 37°C in 5% CO_2 until cells form a monolayer over matrigel.

    Note: Incubation times can vary according to standardization methods described at 4.3.
6. On the experiment day: Prepare and label E0771 cells (mouse breast adenocarcinoma cell line, cells are cultured in same condition as 3B-11 cells):

a. Aspirate the cell culture medium from T75cm^2 flask and wash cells with prewarmed PBS.
b. Add 10ml of pre-warmed serum free DMEM and 1µM Cell Tracker Green CMFDA Dye (Thermo Fisher). Incubate cells for 15min at 37°C in 5% CO_2
c. First check the staining under the microscope.
d. Aspirate the medium and repeat step 4.2.3(a-e) to collect cells.
e. Centrifuge cells at 400g for 5 min. Discard the supernatant and wash cells with 1X PBS.
f. Repeat the centrifuge step. Discard the supernatant. Resuspend the cells in DMEM medium with 0.5% v/v FBS and 4.4nM CSF1. Adjust the cell concentration to 2x10^5 cells. Keep the cells on ice until migration assay is ready to start.

7. Preparation of macrophages
    a. Use BMDMs 7 days after maturation and collect macrophages from tissue culture plates as indicated in section 2.4
    b. Centrifuge macrophages at 400g for 5min, discard the supernatant
    c. Resuspend cells in DMEM complete medium with 4.4nM CSF1 and adjust the concentration to 5x10^5 cells/ml
    d. Aspirate the medium from transwells and make sure endothelial cells, matrigel and transwells are not damaged. Using a sterile tweezer flip the membrane upside down on 24-well plate-lid.
    e. Seed 20µl of macrophage suspension on the underside of the transwell and let the macrophages attach for 15min under the hood at room temperature.

Note: Make sure there are no bubbles and the solution are dispersed evenly on the membrane

f. Place the inserts back into the empty 24 wells and let the cells attach for another 15min in the hood.

Note 1: Since endothelial cells will be attached on the matrigel, they won't get dry during the 30min incubation.

Note 2: Step 6 and step 7 could be initiated at the same time and preparation could be coordinated between the waiting time of each step. However as soon as macrophages are attached to the transwell transmigration assay needs to be started. Tumor cells should be kept on ice until they are ready to be placed on transwells for the transmigration.

8. Move transwells to a 12 well plate with 750 µl of 4.4nM CSF1 and DMEM complete medium.
9. Add 200 µl of tumor cells suspension on top of the transwell.
10. 36 hours later, aspirate the medium from top and bottom chamber and fix transwells with 4% w/v paraformaldehyde (PFA) solution for 15min at room temperature.
11. Remove PFA and wash transwells twice with 1X PBS. To remove non-migrated cells, scrub the upper side of the transwells with a cotton swab.
12. Transfer the wells into a 35mm Mat-Tex glass bottom dish. Using an inverted light microscope equipped with 20X (NA+0.40 air) objective and cooled CCD camera, take 20X images from 6 fields. Quantify the number of migrated cells using Fiji/Image J software. The images from multiple fields can be analyzed in batches. Convert images into a grayscale, and by using the threshold tool, create binary images (positive pixels in black, background white). By using

analyze particle feature, select the average size of a cells and count the number of the cells on the membrane. (If the number of migrated cells per 20X is low, cells could be also counted manually).

13. To obtain more accurate results, the whole transmembrane can be imaged using a whole slide scanner microscope. To block the autofluorescence caused by the transmembrane, use a Matrigel coated membrane that blocks transmission of light between 400 and 700nm (Fluoroblok™, Corning, 354165)
    a. Repeat the same procedure until step 12.
    b. Cut the membranes from the transwell by using a scalpel. Using fine tweezers transfer the membranes onto a glass slide.
    c. Place each membrane on a glass slide with the underside of the transwell membrane facing up. Wash membranes with 1X PBS.
    d. Aspirate PBS buffer and counterstain by adding 100µl of 0.5µg/ml DAPI in PBS. Incubate at room temperature for 10 minutes.
    e. Aspirate the DAPI solution, wash membranes twice with PBS.
    f. Aspirate PBS and mount membranes using mounting medium (Prolong Diamond, Thermo Fisher) and seal with coverslip.
    g. After 24 hours when mounting medium is polymerized and coverslip is stabilized, image the entire membrane using a fluorescent whole slide scanner with 20x magnification. Acquire whole slide fluorescence images within 358nm excitation and 461nm emission for DAPI and 492nm excitation and 517 emission for cell tracker green. (AxioScan Z1 Slide scanner, 20X magnification)
14. Process auto-stitched tile images using an image processor (i.e. Definiens AG, Tissue Studio). Detect cell tracker positive tumor cells by using a nuclear

detection and cell simulation algorithm. Detect DAPI and Cell Tracker positive cells on the entire membrane and run the batch analysis for all samples.

Note: This algorithm can be already built-in to the image processor software such as in Definiens AG, Tissue Studio, or new algorithms could be created using average nucleus/cell size and staining thresholding parameters.

4.3 Standardization methods to obtain endothelial cell monolayer for eTEM assay

The methods described below are used to determine an optimum incubation time for endothelial cells to form a confluent monolayer.

1. Repeat step 4.2 (1-4). Incubate endothelial cells for 24, 48, 72 or 96 hours at 37°C in 5% CO_2. A transwell insert with no cells must also be included.
2. Trans-endothelial resistance:
    a. Replace DMEM complete medium with DMEM 0.5% v/v FBS, 1mM sodium pyruvate, 1% v/v PS.
    b. Two hours later measure the transendothelial resistance using EndOhm-6 chamber and EVOM^2 resistance meter (World Precision Instruments) or equivalent.
    c. Express resistance values (Ω) recorded in function of the effective surface area of the filter membrane (Ωxcm^2)
3. Endothelial permeability:
    a. Repeat step 4.2 (1-4)
    b. Replace the upper and bottom chamber medium with phenol red free-DMEM, 0.5 % v/v FBS, 1mM sodium pyruvate, 1% v/v PS and 100µl/ml

70kDa Rhodamine B Isothiocyanate-Dextran (TRITC), (Sigma-Aldrich) and incubate the cells for 36 hours.

c. Take 100µl of samples from the top and bottom chamber. Dilute samples collected from the top chamber 1:5 in phenol-red free DMEM.
d. Read the fluorescence at emission at 586nm (excitation T 557nm) using a microplate reader.
e. Calculate the TRITC concentration based on a standard curve for TRITC.
f. Determine the optimum incubation time for endothelial cells when no TRITC is detected on the bottom chamber and TER has the highest value compared to the other incubation time.

4.4 2D Coculture Time-Lapse Imaging

1. Preparation of Matrigel coated chamber slides.
    a. Add 300µl of 5mg/ml Matrigel (Corning) in alpha-MEM into each well of Lab Tek II 4-well Chamber Slide (Nunc).
    b. Incubate the slides for 1hr at 37°C.
2. During the incubation time of Step 1b, start preparation and labeling of macrophages:
    a. Prepare bone marrow-derived macrophages as shown in procedure 2.4 (step 3-6).
    b. Replace the medium to pre-warmed serum free alpha-MEM medium with 1.25µm Cell Tracker Orange CMTMR Dye (Thermo Fisher).
    c. Incubate cells for 15min at 37°C in 5% v/v CO_2

    Note: During this incubation time, tumor cells could be prepared (step 3.

d. Isolate the labelled macrophages as described in procedure 2.4 (Step 10)
e. Centrifuge cells at 400g for 5 min. Discard the supernatant and wash cells with 1x PBS.
f. Repeat the centrifuge step. Discard the supernatant. Resuspend the cells in alpha-MEM complete medium with 4.4nM CSF1.

3. Preparation of E0771 cells.
    a. After repeating procedure 4.2.5.2a-e, discard the supernatant and resuspend the cells in alpha-MEM complete medium.

4. Aspirate the medium and add 10^4 E0071 cell and 10^4 BMDMs in total 1mL of alpha-MEM complete medium with 4.4nm CSF1, and gently add to Matrigel coated chamber slides. Let them attach to the plate for 1hr at 37°C in 5% CO_2

5. Use an inverted microscope with 10x magnification, motorized stage, CCD camera sensitive for time-lapse imaging (Axiovert 200, Zeiss) and equipped for microscope cage incubator. Set the incubator for 5% v/v CO_2 and 37°C. Acquire brightfield and fluorescent images every 10 minutes on 10 randomly selected fields.

Note: In the current staining protocol the staining intensity and exposure time is kept minimal to reduce phototoxicity. Any modification of the current protocol requires optimization to keep the cell status healthy.

*6. Pro*cess movies in Fiji/ImageJ software and track macrophage-tumor interaction using a manual-tracking tool in the software. Transfer the data to a spreadsheet and calculate the mean of interaction (in minutes) and the percentage of interacting cells.

Note: The examples of the movies and quantification results are in Kitamura et al., 2015)

Concluding Remarks

In this study we have shown a reproducible macrophage differentiation and characterization method using murine bone marrow derived macrophages and introduced two coculture methods to study macrophage-tumor interactions. Cytokine doses used for macrophage differentiation have been tested and characterized in many other studies (Cho et al., 2014; Zajac et al., 2013; Zhao et al., 2017), however the dose of conditioned medium used for tumor-conditioned macrophages needs to be optimized for each experiment. The confluency of tumor cells, the origin of cell lines, the type of the medium, the batch of FBS and storage conditions might affect the quality of the conditioned medium and macrophage differentiation. To improve the reproducibility, these parameters need to be kept the same between each experiment.

We demonstrated three of the most commonly studied macrophage subtypes *in vitro*. Although macrophages are differentiated through LPS stimulation do not represent a tumor-associated macrophage phenotype but instead a bacteriocidal phenotype, reprogramming alternatively activated macrophages into classically activated macrophages has been shown to promote an anti-tumoral effect and macrophage reprogramming has been studied *in vitro* (Genard, Lucas, & Michiels, 2017). Using LPS or IFNγ stimulation decreases CSF1 receptor level of BMDMs *in vitro* therefore macrophage polarization should be induced after BMDMs are grown in CSF1 rich medium (Stanley, 1985). In addition, BMDMs derived from C3H/HeJ and C57BL/10Scr strains are not responsive to LPS stimulation and this allows for testing of reagents that may be contaminated by endotoxin (Guilbert & Stanley, 1984; Poltorak et al., 1998). It is also possible to use TNF-α rather than IFN-γ for activated macrophage

differentiation (Martinez, Sica, Mantovani, & Locati, 2008). Thus, it is absolutely necessary to define treatments rather than give generic descriptions to macrophage types.

Cytokine mediated differentiation causes changes in expression of a wide variety of cell-surface and intracellular markers, and flow cytometry is a robust method to characterize and monitor those changes. In our study we selected a set of markers that shows the evident differences between M(IFNγ+LPS), M(IL4+IL13) and TCM, however marker choice can be enriched by using other markers (Table 3).
We describe two tumor-macrophage coculture assays that have been validated in our earlier studies. Transmigration models of extravasation could be adapted for tumor intravasation (iTEM) by changing the order of cells seeded in the transwells such that the macrophages and tumor cells are co-cultured together on top of the filter with CSF1 in the lower chamber (Pignatelli et al., 2014). 2D macrophage-tumor coculture assays are a robust method to study the direct interaction of those cells, yet to mimic the complexity of tumor microenvironment more advanced 3D models can be also used (Rebelo et al., 2018).

It is important to note that *in vitro* macrophage differentiation is in fact an oversimplification of the actual *in vivo* situation. Macrophages are highly susceptible to changes within the extracellular environment and they display a wide spectrum of subtypes (Mosser & Edwards, 2008) particularly in the tumor microenvironment (Condeelis & Pollard, 2006). Indeed, the exact mechanisms of *in vivo* differentiation and macrophage ontogeny in some tissues are still not well known and this makes it difficult to define a standard method for macrophage characterization *in vivo*.

Nevertheless *in vitro* macrophage differentiation and *in vitro* co-culture studies continues play an essential role to reveal fundamental mechanisms.

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Tables

Table 1. BMDM Differentiation Reagents

Macrophage subtype	**Reagents**
M(IFNγ+LPS) or 'Activated'	IFN-γ + LPS
M(IL4+IL13) or 'Alternatively activated'	IL-4 + IL-13
Tumor conditioned macrophages (TCM)	20%v/v Tumor-conditioned medium

Table 2. Differentiation Reagents

Differentiation Reagents	**Final Concentration**	**Catalogue Number**	**Supplier**
IFN-γ	20ng/ml	315-05	Peprotech
LPS	100ng/ml	297-473-0	Sigma-Aldrich
IL-13	20ng/ml	210-13	Peprotech
IL-4	20ng/ml	214-14	Peprotech

Table 3. Suggested cell surface markers for identification of BMDM subtype via flow cytometry

BMDM subtype	**Markers**
Pan-macrophage (lineage)	$CD45^+$, $Ly6C^-$, $Ly6G^-$, $F4/80^+$, CD115+ $CD11b^+$
M(IFNγ+LPS) or 'Activated'	$CD80^+$, $CD86^+$, MHC-II^{high}, $TLR\text{-}4^+$

M(IL4+IL13) or 'Alternatively activated' and TCM	$CD206^{+}$, $Arg1^{+}$, $CD163^{+}$, $CD204^{+}$, $CD209^{+}CD169^{+}$ $MHCII^{high/low}$

Table 4. Samples staining and controls for flow cytometry

Tube name	**Samples to be stained**	**Ab's to be used**
Unstained	Cells	No Ab's- just buffer and Fc Block
Full stain	Cells	All Ab's + DAPI
Isotype controls	Cells	All Ab's, swap one Ab for its isotype control instead + DAPI
Fluorescence-minus one controls (FMO)	Cells	All Ab's apart from Ab of interest+ DAPI
Compensation Controls	Beads	Single Ab

Table 5. Antibody list for immunolabeling

Antibody	**Dilution**	**Cat. No.**	**Company**
CD45-PerCPCy5.5	1/100	109827	BioLegend®
Ly6C-BV711	1/100	128037	BioLegend®
Ly6G-BV510	1/200	127633	BioLegend®
CD11b-BV605	1/200	101237	BioLegend®
F4/80-APC	1/50	MCA497-A647	Bio-Rad
CD86-APCCy7	1/100	105029	BioLegend®

MHC-II-FITC	1/00	107605	BioLegend®
CD206-PECy7	1/100	141719	BioLegend®
Isotype ctrl for CD86	1/100	400523	BioLegend®
Isotype ctrl for MHC-II	1/100	400633	BioLegend®
Isotype ctrl for CD206	1/100	40052	BioLegend®

Figure Legends

Figure 1. A. Flow cytometry gating strategy to identify BMDM B. Geometric mean Bar charts and C. Histograms showing the expression of BMDM markers exposed to M(IFNγ+LPS), M(IL4+IL13), TCM polarizing conditions. The color legends are same in B and C. D. Flow cytometry dot plots of M(IFNγ+LPS), M(IL4+IL13), TCM showing the expression of different BMDM markers